

## **Manganese promotes increased formation of hydrogen peroxide by activated human macrophages and neutrophils *in vitro***

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**Running head:** Effects of manganese chloride on human neutrophils.

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## Abstract

Although pro-inflammatory mechanisms have been implicated in the pathogenesis of manganese ( $Mn^{2+}$ )-related neurological and respiratory disorders, relatively little is known about the potential of this metal to interact pro-oxidatively with human phagocytes. The primary objective of the current study was to investigate the effects of  $Mn^{2+}$  as  $MnCl_2$  (0.5-100 $\mu$ M) on the generation of the reactive oxygen species (ROS), superoxide, hydrogen peroxide ( $H_2O_2$ ), and hypohalous acids by isolated human blood neutrophils and monocyte-derived macrophages following activation of these cells with the chemotactic tripeptide, FMLP (1 $\mu$ M), or the phorbol ester, PMA (25ng/ml). Generation of ROS was measured using the combination of oxygen consumption, lucigenin/luminol-enhanced chemiluminescence, spectrofluorimetric detection of oxidation of 2,7-dichlorodihydrofluorescein, radiometric assessment of myeloperoxidase (MPO)-mediated protein iodination, release of MPO by ELISA, and spectrophotometric measurement of nitrite formation. Treatment of activated neutrophils with either FMLP or PMA resulted in significantly decreased reactivity of superoxide in the setting of increased formation of  $H_2O_2$  and MPO-mediated iodination, with no detectable effects on either oxygen consumption or MPO release. Similar effects of the metal with respect to superoxide reactivity and  $H_2O_2$  formation were observed with activated macrophages, while generation of NO was unaffected. Taken together with the findings of experiments using cell-free ROS-generating systems, these observations are compatible with a mechanism whereby  $Mn^{2+}$ , by acting as a superoxide dismutase mimetic, increases the formation of  $H_2O_2$  by

activated phagocytes. If operative *in vivo*, this mechanism may contribute to the toxicity of  $Mn^{2+}$ .

**Key words:** manganese, reactive oxygen species, hydrogen peroxide, neutrophils, macrophage

## Introduction

Manganese ( $Mn^{2+}$ ) is required in trace amounts for normal physiological functions, but can be harmful in the occupational (and also environmental) setting in which individuals are chronically exposed to high levels of the metal.  $Mn^{2+}$  toxicity is associated with the development of manganism, a Parkinson-like neurological disorder, as well as respiratory conditions such as pneumonia, bronchitis and impaired pulmonary function (Akbar et al., 2002; Roth & Garrick, 2003; Takeda, 2003; Cersosimo et al., 2006).

Occupational exposure can occur from the chronic inhalation of  $Mn^{2+}$ -containing fumes and dust associated with mining and ore-grinding, the ferromanganese, iron and steel industries, welding, and in dry-cell battery factories, as well as in the agricultural sector in workers using  $Mn^{2+}$ -based fungicides (Roels et al., 1992; Meco et al., 1994; Bowler et al., 2007; Santamaria, 2008). Environmental exposure may occur in those living in close proximity to mining industries where manganese containing dust can be released into the atmosphere. Concern has

also been raised about possible environmental exposure to manganese via the combustion of methylcyclopentadienyl manganese tricarbonyl (MMT), used as a fuel additive in some countries (Frumkin & Solomon, 1997).

Human polymorphonuclear leukocytes, predominantly neutrophils, as well as macrophages, are crucially involved in the innate host response to infection by phagocytosing, and killing microbial pathogens via an arsenal of toxic molecules such as proteolytic enzymes, reactive oxygen species (ROS), and bacteriocidal proteins (Smith, 1994; Kobayashi, et al., 2005). Although potent, these antimicrobial defenses are indiscriminate and may cause significant inflammation-mediated damage to bystander host tissues. Phagocyte-derived ROS are a group of chemically reactive molecules derived from molecular oxygen generated by the catalytic action of the multicomponent enzyme system, NADPH oxidase. The main members include superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH\cdot$ ) and hypohalous acid. The toxicity of  $H_2O_2$  is enhanced by its reaction with free iron to form highly reactive  $OH\cdot$  (Fenton reaction), or by the activity of myeloperoxidase (MPO). In combination with  $H_2O_2$ , MPO can oxidize the halides to hypohalous acids such as hypochlorous acid, a highly reactive oxidizing agent. Up to 80% of the  $H_2O_2$  generated by activated neutrophils is used to form 20–400  $\mu M$  HOCl an hour, depending on the potency of the stimulus and cell number (Yap et al., 2007).

Exposure to  $Mn^{2+}$  in the environment with subsequent inhalation of particulate matter exposes individuals to both soluble and insoluble forms of  $Mn^{2+}$ . The bioaccessibility of  $Mn^{2+}$  in lung fluids has been investigated in animal models and found to be about 32 – 52% in fluids which closely mimic the lining fluids of the lungs (Julien et al., 2011). Therefore, it is likely that both soluble and insoluble metal components contribute to inhalation-mediated inflammatory responses (Julien et al., 2011). Moreover,  $Mn^{2+}$  accumulation in the central nervous system following inhalation exposure is dependent on particulate solubility (Roels et al., 1997; Dorman et al., 2001), which suggests that the reactivity of the cation is retained during the translocation process. Metals such as  $Mn^{2+}$  induce monocytes/macrophages to release proinflammatory cytokines such as  $TNF-\alpha$ ,  $IL-1\beta$  and  $IL-8$  (Antonini et al., 2003), which are important in mediating transendothelial migration and chemotaxis of neutrophils. We have also observed that  $MnCl_2$  enhances the production of  $IL-6$  and  $IL-8$  by resting and activated monocytes in vitro (unpublished observations). In keeping with these observations, metal extracts from welding fumes have been shown in a rat model to trigger a cytokine response and an influx of neutrophils (Antonini et al., 2003) into the animals' lungs with consequent lung injury. Pulmonary cytotoxicity was confirmed by measuring lactate dehydrogenase and albumin concentrations in bronchoalveolar lavage fluid (Antonini et al., 2003).

Oxidative injury has also been implicated in the pathogenesis of  $Mn^{2+}$ -mediated toxicity (Milatovic et al., 2009). However, the putative molecular/biochemical

mechanisms, including possible interactions of the metal with the ROS-generating neutrophil, remain to be established. The primary aim of the current study was to investigate the effects of  $\text{MnCl}_2$  on the generation of ROS by activated human neutrophils, as well as by human monocyte-derived macrophages in a more limited series of experiments.

## Materials and Methods

### *Chemicals and reagents*

Manganese chloride ( $\text{MnCl}_2$ ) was purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in distilled water to a stock concentration of 10mM, and used in the various assays described below at a final concentration range of 1-100 $\mu\text{M}$ . Unless indicated, all other chemicals and reagents were purchased from Sigma–Aldrich.

### *Neutrophils*

Permission to draw blood from healthy, non-smoking adult human volunteers was granted by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria. Subsequent to obtaining informed consent, neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood and separated from mononuclear leukocytes by centrifugation on

Histopaque-1077 (Sigma-Aldrich) cushions at 400 g for 25 min at room temperature. The resultant cell pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to  $1 \times 10^7$ /ml in PBS and held on ice until used.

#### *Monocyte/macrophage isolation and culture*

Following centrifugation of heparinized whole blood on Histopaque-1077 cushions as described above, the mononuclear leukocyte (MNL) fraction at the plasma/ Histopaque-1077 interface was aspirated, diluted  $\frac{1}{4}$  with PBS and the cells pelleted by centrifugation after which contaminating erythrocytes were removed by hypotonic lysis. Subsequent to another centrifugation step and discarding of the supernatant fluid, the cells were resuspended in sterile Hanks' balanced salt solution (HBSS, indicator-free, containing 1.25mM  $\text{CaCl}_2$ , pH 7.4, Highveld Biological, Johannesburg). The cell preparation was then analysed flow cytometrically using a Beckman Coulter FC500 Flow Cytometer using the following fluorochrome-labelled monoclonal antibodies (Beckman Coulter, Miami, FL, USA): CD3 (FITC), CD14 (PE), CD15 (FITC) and CD19 (PE) for analysis and enumeration of total T cells, monocytes, granulocytes and B-cells, respectively. Differential adherence to plastic was used to separate monocytes from other

types of MNL and to promote their differentiation. MNL (30ml of a  $3 \times 10^7$  MNL/ml suspension in HBSS) were seeded onto sterile 75 cm<sup>3</sup> tissue culture flasks and incubated for 2 hours at 37°C/ 5% CO<sub>2</sub> to promote adherence of monocytes. Following incubation, each flask was gently rinsed with 50 ml of pre-warmed PBS to remove non-adherent cells. Ten milliliters of tissue culture medium RPMI 1640 (Bio Whittaker, Walkersville, MD, USA) supplemented with, antibiotics (penicillin: streptomycin: amphotericin B, 0.1:0.25:0.1 µg/ml) and 5% autologous serum were then added to each flask, which were then incubated for 7 days at 37°C/ 5% CO<sub>2</sub> as described previously (Cassol et al., 2009).

Following the 7-day incubation period, the tissue culture medium was discarded and each flask rinsed once with 10 ml pre-warmed PBS, followed by addition of 10ml PBS containing the Ca<sup>2+</sup>-chelating agent ethylene glycol-bis (2-aminoethylene)-N,N,N,N-tetracetic acid (EGTA, 2mM, final) and the flasks placed on ice with gentle agitation every 10 minutes for at least 30 minutes, to promote detachment of the cells, which were then dislodged by scraping the surface of the flask with a sterile 1.8 x 25 cm Cell Scraper (Adcock Ingram, Scientific Group). The cells were then pelleted by centrifugation, the supernatant discarded and the cell pellet resuspended in 3ml of Ca<sup>2+</sup>-free HBSS containing 2mM EGTA. The cell suspension (350µl) was then analyzed flow cytometrically using the following combination of fluorochrome-labelled monoclonal antibodies: CD14-PE/ CD16-FITC (monocytes/macrophages). The purity of the monocyte/macrophage suspensions (CD14/CD16 dual expressing cells was 83±2%. The remaining



populations consisted of undifferentiated monocytes ( $7\pm 1\%$  expressing CD14 only) and the double negative cells ( $9\pm 1\%$ , probably lymphocytes). The viability of the total cell population measured flow cytometrically by propidium iodide exclusion was  $78\pm 3\%$  (range 62-94%)

These monocyte-derived macrophages were used in the assays of lucigenin chemiluminescence, intracellular  $H_2O_2$  and NO production described below.

#### Measurement of the effects of $Mn^{2+}$ on the generation and reactivities of ROS

When used in combination to measure the generation of ROS by activated neutrophils and cell-free systems, the assays shown in Table 1 not only enable identification of the type of ROS, but also the mechanism involved *i.e.* increased production, conversion of one type of ROS to another, and/or ROS-scavenging activity.

#### *Lucigenin-enhanced chemiluminescence*

Superoxide production was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence method (Minkenberg and Ferber, 1984). Neutrophils ( $1 \times 10^6/ml$ , final) were preincubated for 10min in 900  $\mu l$  indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM  $CaCl_2$ ) containing 0.2 mM lucigenin in the presence and absence of  $MnCl_2$  (0.1-100 $\mu M$ ). The reaction mixtures were then stimulated with either the phorbol ester, PMA (phorbol 12-

myristate 13-acetate; 25ng/ml final) or the synthetic chemoattractant, N-formyl-L-leucyl-L-phenylalanine (FMLP, 1 $\mu$ M final), and chemiluminescence responses recorded in an LKB Wallac chemiluminometer (Turku, Finland) for PMA-activated systems or a Lumac Biocounter 2010 for FMLP-stimulated systems. The results are expressed as the peak responses in mV/sec and relative light units for PMA- and FMLP-activated systems respectively. These peak responses occurred at 30-60sec (FMLP) and 5min (PMA).

The effects of MnCl<sub>2</sub> (50-400 $\mu$ M) on lucigenin-enhanced chemiluminescence were also determined using PMA activated monocytes/macrophages (3x 10<sup>5</sup>cells/ml) suspended in Ca<sup>2+</sup>-replete HBSS. The set-up of these experiments was similar to that described above for neutrophils and the results expressed as percentage of the Mn<sup>2+</sup>-free control system.

Additional experiments were undertaken to determine the potential of MnCl<sub>2</sub> to scavenge superoxide, using a cell-free, xanthine/xanthine oxidase superoxide-generating system. Reaction mixtures consisted of xanthine oxidase (64mU/ml), lucigenin (0.2 mM) and xanthine (0.9mM) in the presence or absence of MnCl<sub>2</sub> (6.25-100 $\mu$ M) in a final volume of 1ml HBSS. Results are expressed as the peak chemiluminescence responses which occurred after approximately 14 min.

### *Oxygen Consumption*

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils ( $2 \times 10^6$ ) were preincubated for 10 min at 37°C in HBSS in the presence or absence of  $\text{MnCl}_2$  (25 $\mu\text{M}$ ) followed by activation of the cells with PMA (25ng/ml).  $\text{PO}_2$  was monitored for a further 10 minutes. Oxygen consumption was measured over the period when consumption was linear, which was 7 min, and the results expressed as nmol/min/ $2 \times 10^6$  cells.

### *Luminol-enhanced chemiluminescence*

Luminol-(5-amino-2,3-dihydro-1,4-phthalazine dione) enhanced CL, which detects hydroxyl radical, (Yildiz G & Demiryürek, 1998) was used in the following cell-free experiments to determine: i) the potential of  $\text{MnCl}_2$  to generate hydroxyl radical from  $\text{H}_2\text{O}_2$  by a Fenton type reaction; and ii) the effects of  $\text{MnCl}_2$  on the generation of hydroxyl radical in a Fenton reaction involving the interaction of vanadium (III) chloride with  $\text{H}_2\text{O}_2$  (Fickl et al., 2006). In the case of the former, reaction systems contained luminol (0.1mM), glucose, (5mM in HBSS) and glucose oxidase (400mU/ml from *Aspergillus niger*) without and with  $\text{MnCl}_2$  (25 $\mu\text{M}$ ) in a final reaction volume of 1 ml. The latter system also contained luminol, glucose and glucose oxidase, as well as vanadium (III) chloride (25 $\mu\text{M}$ ) without and with  $\text{MnCl}_2$  and the hydroxyl scavenger, mannitol (20mM). In both

experimental systems the reactions were initiated by the addition of glucose oxidase, and luminol-enhanced chemiluminescence measured using the LKB Wallac 1251 chemiluminometer as described above.

### *Intracellular H<sub>2</sub>O<sub>2</sub> production*

Intracellular H<sub>2</sub>O<sub>2</sub> was measured using a 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA)-based spectrofluorimetric procedure (Rhee et al., 2010). This agent is hydrolyzed by cellular esterases to 2',7'-dichlorodihydrofluorescein which is oxidized to 2',7'-dichlorofluorescein primarily by H<sub>2</sub>O<sub>2</sub>. DCF-DA (1μM) was added to a reaction mixture containing 1x10<sup>6</sup>/ml neutrophils or 1x10<sup>5</sup>/ml monocyte-derived macrophages in the absence and presence of MnCl<sub>2</sub> (3-25μM) in a total volume of 3 ml HBSS. The samples were then incubated for 10 min in a 37°C waterbath, then transferred to the thermoregulated cuvette holder of a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 500 and 530nm respectively. Baseline fluorescence responses and those activated with FMLP (1μM) or PMA (25ng/ml) were then monitored for 10min. These experiments were performed in the presence and absence of the MPO inhibitors sodium azide (0.7mM) or 4-aminobenzoic acid hydrazide (ABAH, 50μM) to eliminate the complicating effects of oxidation by hypochlorous acid (Kettle et al., 1997; Tintinger et al., 2007). These results are shown as the traces of representative experiments.

The validity of using DCF-DA as an oxidizable substrate for the spectrofluorimetric detection of H<sub>2</sub>O<sub>2</sub> was confirmed in a series of preliminary experiments using a cell-free H<sub>2</sub>O<sub>2</sub>-generating system (glucose/glucose oxidase), which clearly demonstrated an intense increase in fluorescence intensity (data not shown).

#### *MPO-mediated protein iodination*

This was performed according to the method of Root and Stossel with minor modifications (Root & Stossel, 1974). Neutrophils were preincubated for 10 min at 37°C in 900µl of HBSS containing 1 µCi of iodine-125 (as Na<sup>125</sup>I, 37MBq, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), 20 µM cold carrier NaI and 2mg/ml of bovine serum albumin (BSA) in the presence and absence of MnCl<sub>2</sub> (0.5-100µM). The cells were then activated by addition of either FMLP (1µM) or PMA (25ng/ml) and incubated for 10min at 37°C after which the protein in the reaction mixtures was precipitated by addition of 20% trichloroacetic acid (TCA) and the precipitates pelleted by centrifugation, followed by 3 more wash steps with TCA to remove unbound <sup>125</sup>I. The amount of protein-associated <sup>125</sup>I in the precipitates was determined using a Perkin Elmer 2470 Automatic Gamma Counter and the results expressed pmols <sup>125</sup>I per 2X10<sup>6</sup> cells.

The following additional experiments were performed to determine: i) the effects of DPI (diphenyleneiodonium chloride-10µM), an inhibitor of NADPH oxidase, or

sodium azide (0.7mM), on modulation of MPO-mediated iodination by  $\text{MnCl}_2$ ; ii) the effects of  $\text{MnCl}_2$  at a fixed concentration of  $50\mu\text{M}$  on MPO-mediated protein iodination in a cell-free system containing 50mU/ml of purified MPO (from human leukocytes),  $\text{Na}^{125}\text{I}$  ( $2\mu\text{Ci}$ ), 2mg/ml BSA and glucose (5mM in HBSS)/glucose oxidase (1.5U/ml) as a source of  $\text{H}_2\text{O}_2$  in a final reaction volume of 1ml.

Reactions were terminated and BSA precipitated after 10min of incubation and the protein precipitates processed and analysed as above.

#### *MPO release*

MPO was measured in the supernatants of neutrophils activated with FMLP ( $1\mu\text{M}$ )/cytochalasinB ( $1\mu\text{M}$ , added to enhance degranulation), or PMA (25ng/ml), in the absence and presence of  $50\mu\text{M}$   $\text{MnCl}_2$ . Neutrophils ( $2 \times 10^6/\text{ml}$ , final) in HBSS were preincubated for 10min at  $37^\circ\text{C}$  with  $\text{MnCl}_2$  after which the cells were activated and the reaction mixtures incubated for a further 10 min at  $37^\circ\text{C}$ . The tubes were then transferred to an ice-bath, followed by centrifugation at 400 g for 5min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for MPO using a double-antibody, capture ELISA procedure (Kamiya Biomedical Company, Seattle, WA, USA).

### *Nitric oxide production*

For these experiments, monocyte-derived macrophages suspended in HBSS were added to the wells of micro-tissue culture plates ( $10^5$  cells/well, final volume 200 $\mu$ l) and incubated for 2 hours at 37°C/ 5% CO<sub>2</sub> to promote adherence, after which the HBSS was replaced with serum supplemented RPMI 1640 and the plates incubated overnight at 37°C/ 5% CO<sub>2</sub>. The next day the RPMI 1640/ serum medium in each well was replaced with HBSS without and with MnCl<sub>2</sub> (100 $\mu$ M) followed 30 min later by the macrophage activator, bacterial lipopolysaccharide (from *Escherichia coli* 0127:B8, 1 $\mu$ g/well, final). The plates were then incubated for 24 hours at 37°C/ 5% CO<sub>2</sub> after which the cell-free supernatants were assayed spectrophotometrically for nitrite as a surrogate for NO using the Calbiochem Colorimetric Nitric Oxide Kit (Calbiochem - EMD4 Biosciences, San Diego, CA, USA). Using this procedure, nitrate, also a product of NO, is converted to nitrite by the addition of nitrate reductase. Total nitrite is then determined spectrophotometrically using the Greiss reagent at a wavelength of 540nm and the results expressed as nmol nitrite/ $10^5$  cells.

### *Cellular ATP levels*

To determine the effects of MnCl<sub>2</sub> (100 $\mu$ M) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates ( $2 \times 10^6$  cells/ml) following

exposure of the cells to the metal for 10 min using a luciferin/luciferase chemiluminescence procedure (Holmsen et al., 1972).

#### *Statistical analysis of data*

With respect to organization of data in the “Results” section, these are grouped according to the assays mentioned in the “Materials and Methods” section. In the cases of MPO-mediated iodination and MPO release, only data for neutrophils are shown because of the absence of this enzyme in macrophages, while in the case of nitric oxide production only data for activated macrophages are shown. The results of each series of experiments are presented as the mean values  $\pm$  SEM, either as the absolute values or as mean percentages of the corresponding metal-free control systems, where  $n$  = the number of different donors used in each series of experiments. Levels of statistical significance were determined by comparing the absolute values for each drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using the Wilcoxon matched-pairs signed-ranks test.

Results:

#### *Lucigenin-enhanced chemiluminescence*

The effects of  $\text{MnCl}_2$  on superoxide production by neutrophils activated with FMLP ( $1\mu\text{M}$ ) or PMA (25ng/ml) are shown in Figure 1.  $\text{MnCl}_2$  at concentrations



of 0.1-100 $\mu$ M and 1.5-100 $\mu$ M in the case of FMLP - and PMA-activated cells, respectively, caused significant dose-related inhibition of the lucigenin-enhanced CL responses. In the case of macrophages, inhibitory effects on chemiluminescence were only seen at higher concentrations of MnCl<sub>2</sub> and the results are shown in Figure 2.

The cell-free xanthine/xanthine oxidase superoxide-generating system was used to assess the superoxide-scavenging potential of MnCl<sub>2</sub>. These results are shown in Figure 3 which demonstrate dose-dependent, statistically significant inhibition of chemiluminescence in the presence of the metal. (\*P<0.05 for comparison for each concentration in comparison with the MnCl<sub>2</sub>-free control system; data from 8 experiments).

### *Oxygen Consumption*

Activation of neutrophils with PMA was accompanied by a marked increase in oxygen consumption by the cells that was sustained over a 5-10min period and unaffected by MnCl<sub>2</sub> (25 $\mu$ M). The results for the control PMA-stimulated systems and those treated with 25 $\mu$ M of MnCl<sub>2</sub> were 64 $\pm$ 4 and 62 $\pm$ 6 nmol O<sub>2</sub> consumed/min/2 x 10<sup>6</sup> cells, respectively (n=6). The corresponding values for unstimulated cells in the presence and absence of the metal were 21 $\pm$  2 and 22 $\pm$ 2 nmol/min/2x10<sup>6</sup> cells. These results clearly demonstrate that Mn<sup>2+</sup> does not activate NADPH oxidase. Likewise oxygen consumption by the xanthine/xanthine

oxidase system was unaffected by  $\text{MnCl}_2$ , excluding possible inhibitory effects on the oxidase. Results for oxygen consumption by the xanthine/xanthine oxidase control and  $\text{MnCl}_2$  (25 $\mu\text{M}$ )-treated systems were  $86\pm 2$  and  $86\pm 6$  nmol  $\text{O}_2$  consumed/min respectively (data from 3 experiments).

Taken together these results are compatible with superoxide dismutase mimetic activity of  $\text{MnCl}_2$  in both the neutrophils and xanthine oxidase system.

#### *Luminol-enhanced chemiluminescence*

.The cell-free, glucose/glucose oxidase,  $\text{H}_2\text{O}_2$ -producing, luminol-enhanced chemiluminescence system was used to assess the potential of  $\text{MnCl}_2$  to: i) initiate hydroxyl radical generation via a Fenton-type reaction; and ii) to scavenge hydroxyl radical generated via a Fenton-type reaction (the interaction of vanadium (III) chloride (25 $\mu\text{M}$ ) with  $\text{H}_2\text{O}_2$ ). Luminol-enhanced chemiluminescence values for the control, glucose/glucose oxidase containing systems and those treated with 25 $\mu\text{M}$   $\text{MnCl}_2$  were negligible, being  $11\pm 2$  and  $10 \pm 3$  mV/sec respectively, demonstrating lack of reactivity of  $\text{H}_2\text{O}_2$  with luminol, as well as  $\text{H}_2\text{O}_2$  with  $\text{MnCl}_2$ . Addition of vanadium III to the  $\text{H}_2\text{O}_2$  producing systems, however, resulted in a significant increase in chemiluminescence with a peak response of  $2142\pm 235$  mV/sec ( $P=0.008$  for comparison with the vanadium-free control system); inclusion of  $\text{MnCl}_2$  significantly ( $P=0.008$ ) attenuated the chemiluminescence signal generated by the vanadium III/ $\text{H}_2\text{O}_2$  interaction which decreased to  $175\pm 36$  mV/sec (data from 8 experiments). Importantly, the

vanadium/H<sub>2</sub>O<sub>2</sub> chemiluminescence signal was also attenuated by the traditional hydroxyl radical scavenger mannitol (20mM). Luminol-enhanced CL values for the control, glucose/glucose oxidase systems and those treated with vanadium only or vanadium + mannitol were 37±7, 3107±411 and 1074±206 mV/sec respectively (data from 5 experiments).

Taken together, these results demonstrate that under our experimental conditions MnCl<sub>2</sub> does not interact with H<sub>2</sub>O<sub>2</sub> to generate hydroxyl radical, but rather appears to function as a scavenger of this potent ROS.

#### *Intracellular H<sub>2</sub>O<sub>2</sub> production*

The results shown in Figure 4 are typical traces from one representative experiment (n=4) which depict the effects of MnCl<sub>2</sub> (3-25µM) on the DCF-DA fluorescence responses of FMLP (1µM) - and PMA (25ng/ml)-activated neutrophils in the presence of sodium azide. Treatment of the cells with MnCl<sub>2</sub> caused a dose-dependent increase in fluorescence intensity compatible with augmentation of intracellular H<sub>2</sub>O<sub>2</sub> production by both FMLP and PMA-activated neutrophils. PMA-activated cells treated with MnCl<sub>2</sub> in the presence of ABAH showed similar trends (results not shown). As shown in Figure 5, treatment of macrophages with 25µM MnCl<sub>2</sub> also resulted in significant augmentation of intracellular H<sub>2</sub>O<sub>2</sub> production.

### *MPO-mediated protein iodination*

The effects of  $\text{MnCl}_2$  (0.5-100 $\mu\text{M}$ ) on the activity of the MPO/ $\text{H}_2\text{O}_2$ /halide system of FMLP- or PMA-activated neutrophils, are shown in Figure 6.  $\text{MnCl}_2$  at concentrations of 0.5 $\mu\text{M}$  and higher, caused significant, dose-related enhancement of neutrophil MPO-mediated iodination of BSA following activation of the cells with either FMLP or PMA. In the case of FMLP-activated cells, inclusion of  $\text{MnCl}_2$  at 0.5-100 $\mu\text{M}$  resulted in 19-146% enhancement of MPO-mediated iodination, while with PMA 19%-65% enhancement was noted with metal concentrations of 0.5-12.5 $\mu\text{M}$ , reaching a plateau thereafter.

The stimulatory effects of  $\text{MnCl}_2$  (25 $\mu\text{M}$ ) on FMLP-activated MPO-mediated protein iodination were significantly attenuated by inclusion of DPI (10 $\mu\text{M}$ ) or sodium azide (1mM). The results for unstimulated cells, the FMLP-activated control system, and systems treated with  $\text{MnCl}_2$  only,  $\text{MnCl}_2$  + DPI, or  $\text{MnCl}_2$  + sodium azide, were:  $18 \pm 5$ ,  $214 \pm 39$ ,  $465 \pm 86$ ,  $0.13 \pm 0.07^*$  and  $141 \pm 49^*$  pmol  $^{125}\text{I}/2 \times 10^6$  cells, respectively. (\* $P < 0.05$  for comparison with  $\text{MnCl}_2$  only systems).

The effects of  $\text{MnCl}_2$  (50 $\mu\text{M}$ ) on the iodination of BSA by a cell-free MPO + glucose/glucose oxidase +  $^{125}\text{I}$  system were also evaluated.  $\text{MnCl}_2$  did not significantly affect protein iodination by the cell-free MPO/ $\text{H}_2\text{O}_2$ / $^{125}\text{I}$  system. Values for the systems with and without  $\text{MnCl}_2$  were  $417 \pm 29$ , and  $473 \pm 42$  pmol  $^{125}\text{I}/2\text{mg}$  BSA respectively, while the corresponding values for the background

control systems (without glucose oxidase) were  $94\pm 9$  and  $86\pm 5$  pmol  $^{125}\text{I}/2\text{mg}$  BSA.

#### *MPO release*

MPO release from neutrophils activated with FMLP/cytochalasin B (F/CB) or PMA was not significantly affected by  $\text{MnCl}_2$ . The results for the unstimulated cells, the F/CB activated control system, and systems treated with  $50\mu\text{M}$   $\text{MnCl}_2$  were  $146\pm 17$ ,  $1243\pm 133$  and  $1160\pm 111$  ng/ml MPO, respectively. The corresponding results for the PMA-activated control system and those treated with  $50\mu\text{M}$   $\text{MnCl}_2$  were  $548\pm 50$  and  $510\pm 43$  ng/ml MPO, respectively (n=6).

#### *Nitric oxide production*

The respective values for production of NO by control monocyte-derived macrophages and those treated with  $100\mu\text{M}$   $\text{MnCl}_2$  were  $6.2\pm 0.8$  and  $6.2\pm 0.8$  nmols/ $10^5$  cells respectively. The corresponding values for LPS-activated control cells and those treated with  $100\mu\text{M}$  of  $\text{MnCl}_2$  were  $9.6\pm 0.8$  and  $6.6\pm 0.8$  nmols/ $10^5$  cells (n=5 with 2 replicates for each system in each experiment; no significant differences were detected with respect to comparison of the control and  $\text{MnCl}_2$ -treated systems).

#### *Cellular ATP levels*

Exposure of neutrophils to  $\text{MnCl}_2$  ( $100\mu\text{M}$ ) for 10 min did not significantly affect cellular ATP levels, demonstrating lack of cytotoxicity of the metal at the concentrations and experimental conditions in which these cells were used. The values for control cells and those exposed to  $\text{MnCl}_2$  at concentrations of  $100\mu\text{M}$  were:  $211 \pm 23$  and  $204 \pm 20$  pmols ATP/ $2 \times 10^7$  cells, respectively (n=4, with 3-6 replicates for each system.) Similar results were found using a flow cytometric, propidium iodide dye exclusion procedure, the mean percentages viability for the control and metal-treated ( $50\mu\text{M}$ ) systems being  $99.4 \pm 0.1\%$  and  $99.5 \pm 0.1\%$  respectively.

### *Discussion*

The results of the current study have demonstrated that  $\text{Mn}^{2+}$  potentiates the production of  $\text{H}_2\text{O}_2$  by human neutrophils and macrophages. Somewhat paradoxically, these pro-oxidative interactions of  $\text{Mn}^{2+}$  with human phagocytes are a consequence of the superoxide dismutase mimetic activity of the metal. While  $\text{H}_2\text{O}_2$  *per se* may predispose to oxidant-mediated tissue damage, the toxicity of this ROS is enhanced via its transformation to hypohalous acids by neutrophils and monocytes (Klebanoff et al., 1993).

The SOD mimetic activity of  $\text{Mn}^{2+}$  was documented in an initial series of experiments in which co-incubation of activated human neutrophils and monocyte-derived macrophages with the metal resulted in dose-dependent

inhibition of lucigenin-enhanced chemiluminescence. Relative to neutrophils, somewhat higher concentrations of  $Mn^{2+}$  were required to cause significant inhibition of the chemiluminescence responses of activated macrophages, possibly as a consequence of the absence of MPO in these cells, which was confirmed in the current study (not shown). MPO is a negative regulator of superoxide production by activated neutrophils and monocytes (Locksley et al., 1983, Nauseef et al., 1983). The following lines of evidence confirmed that  $Mn^{2+}$  neutralizes superoxide as opposed to being an inhibitor of its generation by activated phagocytes: i) similar effects to those observed with activated neutrophils and macrophages were observed using a cell-free xanthine/xanthine oxidase superoxide-generating system; ii) the metal did not affect oxygen utilization by either the phagocyte NADPH oxidase or xanthine oxidase; and iii) the production of NO, which in excess neutralizes superoxide (Cauwels et al., 2005), was unaffected by treatment of macrophages with  $Mn^{2+}$ . These neutralizing interactions of  $Mn^{2+}$  with the superoxide anion have been described in several previous studies (Klebanoff et al., 1993; Mackenzie and Martin, 1998; Hussain and Ali, 1999). Although  $Mn^{2+}$  was also found to neutralize hydroxyl radical in a cell-free system, the relevance of this observation in the pathophysiological setting is doubtful, as the production of this highly toxic ROS by phagocytes is stringently controlled by metal-binding proteins.

The effects of  $Mn^{2+}$ , at non-cytotoxic concentrations, on intracellular  $H_2O_2$  concentrations in activated neutrophils and macrophages were investigated using

DCF-DA, a fluorescent dye which emits light when oxidised by  $H_2O_2$ , and to a lesser extent with MPO-derived hypochlorous acid (Gomes et al., 2005; Rhee et al., 2010). When using neutrophils, which contain high concentrations of MPO, these experiments were performed in the absence and presence of the MPO inhibitors, sodium azide and ABAH, to control for the complicating effects of oxidation of DCF by hypochlorous acid. Activation of DCF-DA-loaded neutrophils by FMLP or PMA resulted in marked increases in fluorescence intensity which were considerably greater in the presence of  $Mn^{2+}$ . Importantly, these effects were evident in the presence of sodium azide or ABAH, compatible with increased intracellular concentrations of  $H_2O_2$ . Similar effects were observed with PMA-activated monocyte-derived macrophages at concentrations of  $Mn^{2+}$  equivalent to those used in the neutrophil experiments, albeit in the absence of sodium azide/ABAH as these cells do not contain MPO.

These experiments were extended to investigate the effects of  $Mn^{2+}$  on the generation of hypohalous acids following activation of the neutrophils by FMLP or PMA. Inclusion of  $Mn^{2+}$  caused significant dose-related enhancement of the iodination of added protein, which was attenuated by the inclusion of inhibitors of NADPH oxidase or MPO. The following lines of evidence implicated increased formation of  $H_2O_2$  via superoxide dismutase mimetic activity as the mechanism of  $Mn^{2+}$ -mediated increase in iodination of proteins by activated neutrophils: i) these effects of  $Mn^{2+}$  were not observed in a cell-free system consisting of purified



human MPO, H<sub>2</sub>O<sub>2</sub> and <sup>125</sup>I; ii) there was no detectable increase in the release of MPO from activated neutrophils in the presence of Mn<sup>2+</sup>.

With the exception of an earlier study by Klebanoff *et al.* (1993), the pro-oxidative interactions of Mn<sup>2+</sup> with human phagocytes and their possible involvement in the pathogenesis of occupation-related neurological and respiratory disorders are largely under-appreciated. Klebanoff and colleagues (1993) also concluded that Mn<sup>2+</sup>, by acting as a superoxide dismutase mimetic, resulted in increased accumulation of H<sub>2</sub>O<sub>2</sub> by activated phagocytes. However, these investigators used a scopoletin-based spectrofluorimetric procedure which does not distinguish between H<sub>2</sub>O<sub>2</sub> and hypochlorous acid, as opposed to the DCF-DA-based method used in the current study. Notwithstanding efforts to exclude effects of Mn<sup>2+</sup> on the release and activity of MPO, other important distinctions between the two studies include our findings that Mn<sup>2+</sup> does not affect the activity of NADPH oxidase, and, most importantly, that the metal also interacts pro-oxidatively with human monocyte-derived macrophages. We do concede, however, that the effects of Mn<sup>2+</sup> with these cells may not be entirely representative of the interactions of the metal with alveolar macrophages.

Although SOD mimetics can be protective by scavenging superoxide radicals and attenuating oxidative stress (Vuokko and Crapo, 2003), these agents have the potential to induce tissue injury at higher concentrations (McCord and Edeas, 2005). A bell-shaped dose-response curve exists for superoxide dismutases with

the protective effect being lost above a threshold concentration (McCord and Edeas, 2005). Multiple mechanisms for the increase in tissue injury have been proposed, including an increase in H<sub>2</sub>O<sub>2</sub> concentrations (Omar et al., 1990). However, irrespective of the mechanism/s involved, SOD mimetics may exacerbate tissue damage (Ye et al., 2011; Batinic-Haberle et al., 2011).

Blood levels of Mn<sup>2+</sup> have been reported to range from 4-12 µg/L (73-210 nmol/L) in healthy individuals (Reynolds *et al.*, 1994), reaching up to 17.3 µg/L in individuals occupationally exposed to high atmospheric levels of the metal (Smith et al., 2007). Although somewhat lower than the threshold concentration of Mn<sup>2+</sup> at which augmentation of intracellular H<sub>2</sub>O<sub>2</sub> concentrations were observed in activated neutrophils/macrophages (0.5 µM=27.5 ng/ml Mn<sup>2+</sup>), it is noteworthy that blood levels of the metal do not reflect those of cells and tissues, which are considerably higher (Smith et al., 2007; Finkelstein et al., 2008; Pejović-Milić et al., 2009). Store-operated calcium channels, as well as other mechanisms operative at the blood-brain barrier are likely to promote cellular uptake of Mn<sup>2+</sup> (Crossgrove and Yokel, 2005). Our study is potentially limited by an absence of data on the long-term effects of Mn<sup>2+</sup> deposition within tissues.

In humans, the respiratory tract represents the primary route of access of Mn<sup>2+</sup>, predisposing in the occupational setting to the inflammatory airway disorders bronchitis and pneumonitis, as well as subacute bronchiolitis in experimentally-exposed rhesus monkeys (Dorman et al., 2005). Inhaled Mn<sup>2+</sup> enters the

bloodstream and accumulates in the central nervous system (Roels et al., 1997; Dorman et al., 2001) where it interacts with tissue macrophages (microglial cells) to potentiate lipopolysaccharide/interferon- $\gamma$ - induced TNF- $\alpha$  gene expression (Chen et al., 2006). Activated glial cells release pro-inflammatory cytokines (Filipov et al., 2005), mediators and reactive oxidants (Chen et al., 2006), which may injure adjacent neurons (Minghetti and Levi, 1998) and predispose to neurodegenerative disorders (Hirsch, 2000). These pro-inflammatory effects of the metal appear to result from oxidative activation of the transcription factor NF kappa B (Barhoumi et al., 2004; Filipov et al., 2005). These effects are summarized in Figure 7.

Phagocyte-derived ROS are potent cytotoxic and pro-inflammatory agents that directly oxidize critical protein sulfhydryls, iron-sulfur centers and heme moieties, and react with amines to form chloramines (Goud et al., 2008). In addition, H<sub>2</sub>O<sub>2</sub> is a well-recognized activator of intracellular signalling mechanisms, promoting oxidative activation of transcription factors such as nuclear factor  $\kappa$ -B and Ca<sup>2+</sup> influx in various types of immune and inflammatory cells, creating a highly pro-inflammatory environment (Schmidt et al., 1995; Takada et al., 2003; Giambelluca and Gende, 2008).

In conclusion, Mn<sup>2+</sup>, at concentrations which may be relevant in the setting of occupational exposure to the metal, increases the formation of H<sub>2</sub>O<sub>2</sub> by activated neutrophils and macrophages. If not counteracted by the superoxide- and

hydroxyl radical-neutralizing activities of  $Mn^{2+}$ , the pro-oxidative interactions of the metal with phagocytes may contribute to the pathogenesis of  $Mn^{2+}$ -mediated respiratory and neurological disorders.

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Table 1 Strategies used to identify the effects of manganese on the generation of ROS by activated neutrophils and cell-free enzymatic systems, as well as on the reactivities of these oxidants.

| Assay                                      | Application using:                                         |                                                                                                                                                                                                              |
|--------------------------------------------|------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                            | Activated Neutrophils                                      | Cell-free systems                                                                                                                                                                                            |
| Lucigenin-enhanced CL*                     | Primarily detects superoxide production                    | To detect superoxide scavenging activity using the xanthine/xanthine oxidase system                                                                                                                          |
| Luminol-enhanced CL                        | -                                                          | To monitor hydroxyl radical-scavenging activity using a glucose/glucose/vanadium(3 <sup>+</sup> ) system                                                                                                     |
| Dichlorofluorescein diacetate fluorescence | Detects intracellular hydrogen peroxide                    | -                                                                                                                                                                                                            |
| MPO <sup>+</sup> -mediated iodination      | Detects hypohalous acids                                   | To determine effects on MPO, as well as hypohalous acid scavenging activity using a system with added MPO, <sup>125</sup> I, bovine serum albumin, and glucose/glucose oxidase to generate hydrogen peroxide |
| Oxygen Consumption                         | To measure effects on activation/activity of NADPH oxidase | To validate superoxide-scavenging activity using the xanthine/xanthine oxidase system                                                                                                                        |

\* CL= chemiluminescence; + MPO=myeloperoxidase

## Legends to Figures

### Figure 1

Effects of  $\text{MnCl}_2$  (0.1-100 $\mu\text{M}$ ) on the lucigenin-enhanced chemiluminescence responses of neutrophils activated by FMLP (1 $\mu\text{M}$ ) and PMA (25ng/ml). The results are expressed as the mean peak chemiluminescence values in relative light units (FMLP systems) and mV/sec (PMA systems)  $\pm$ SEM (n=5 with 2-5 replicates for each system). The absolute values for the unstimulated and FMLP activated systems and those for the unstimulated and PMA-activated systems were  $3453 \pm 1290$ ,  $6272 \pm 763$  relative light units and  $623 \pm 94$ ,  $4742 \pm 284$  mV/sec, respectively. \*P<0.05 for comparison with the  $\text{MnCl}_2$ -free control system.

### Figure 2

Effects of  $\text{MnCl}_2$  (50-400 $\mu\text{M}$ ) on the lucigenin-enhanced chemiluminescence responses of macrophages activated by PMA (25ng/ml). The results are expressed as the mean peak chemiluminescence values in mV/sec  $\pm$  SEM (n=5). The absolute values for the unstimulated and PMA-activated systems were  $557 \pm 177$ ,  $2952 \pm 842$  mV/sec, respectively. \*P<0.05 for comparison with the  $\text{MnCl}_2$ -free control system.

### Figure 3

Effects of  $\text{MnCl}_2$  (6.25-100 $\mu\text{M}$ ) on the lucigenin-enhanced chemiluminescence by the cell-free, xanthine/xanthine oxidase superoxide-generating system. The results are expressed as the mean peak chemiluminescence values in  $\text{mV}/\text{sec} \pm \text{SEM}$  (8 replicates). \* $P < 0.05$  for comparison with the  $\text{MnCl}_2$ -free control system.

### Figure 4

Traces from a single representative experiment ( $n=4$ ) showing the effects of  $\text{MnCl}_2$  at concentrations of 3-25 $\mu\text{M}$  on the dichlorofluorescein diacetate fluorescence responses of FMLP(1 $\mu\text{M}$ )- and PMA (25 $\text{ng}/\text{ml}$ )-activated neutrophils. FMLP and PMA were added as indicated ( $\downarrow$ ) after a stable baseline was obtained.

Figure 5. PMA-activated dichlorofluorescein fluorescence responses in macrophages activated with PMA (25 $\text{ng}/\text{ml}$ ) in the absence (—) and presence (----) of 25 $\mu\text{M}$  of  $\text{MnCl}_2$ . PMA was added as indicated ( $\downarrow$ ) after a stable baseline was obtained. These are 3 typical traces from 4 experiments using cells from 4 different donors.

## Figure 6

Effects of  $\text{MnCl}_2$  (0.5-100 $\mu\text{M}$ ) on MPO-mediated iodination of protein by neutrophils activated with FMLP (1 $\mu\text{M}$ ) or PMA (25ng/ml). The results are expressed as the mean values in  $\text{pmol } ^{125}\text{I}/2 \times 10^6 \text{ cells} \pm \text{SEM}$  (n=6 with triplicate values for each system). The absolute values for MPO-mediated iodination for unstimulated, FMLP- and PMA activated systems were,  $14 \pm 4$ ,  $168 \pm 13$  and  $2354 \pm 258 \text{ pmol } ^{125}\text{I}/2 \times 10^6 \text{ cells}$ , respectively. \* $P < 0.05$  for comparison with the  $\text{MnCl}_2$ -free control system.

## Figure 7

Neutrophil membrane-associated NADPH oxidase generates superoxide anions ( $\text{O}_2^-$ ) which in the presence of  $\text{Mn}^{2+}$ , an SOD mimetic, is converted to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).  $\text{H}_2\text{O}_2$  activates NF- $\kappa\text{B}$  and promotes the synthesis of pro-inflammatory cytokines and is also transformed to HOCl by myeloperoxidase (MPO) released from primary granules. Both HOCl and pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- $\alpha$  contribute to neutrophil-mediated inflammation and tissue injury.



Figure 1

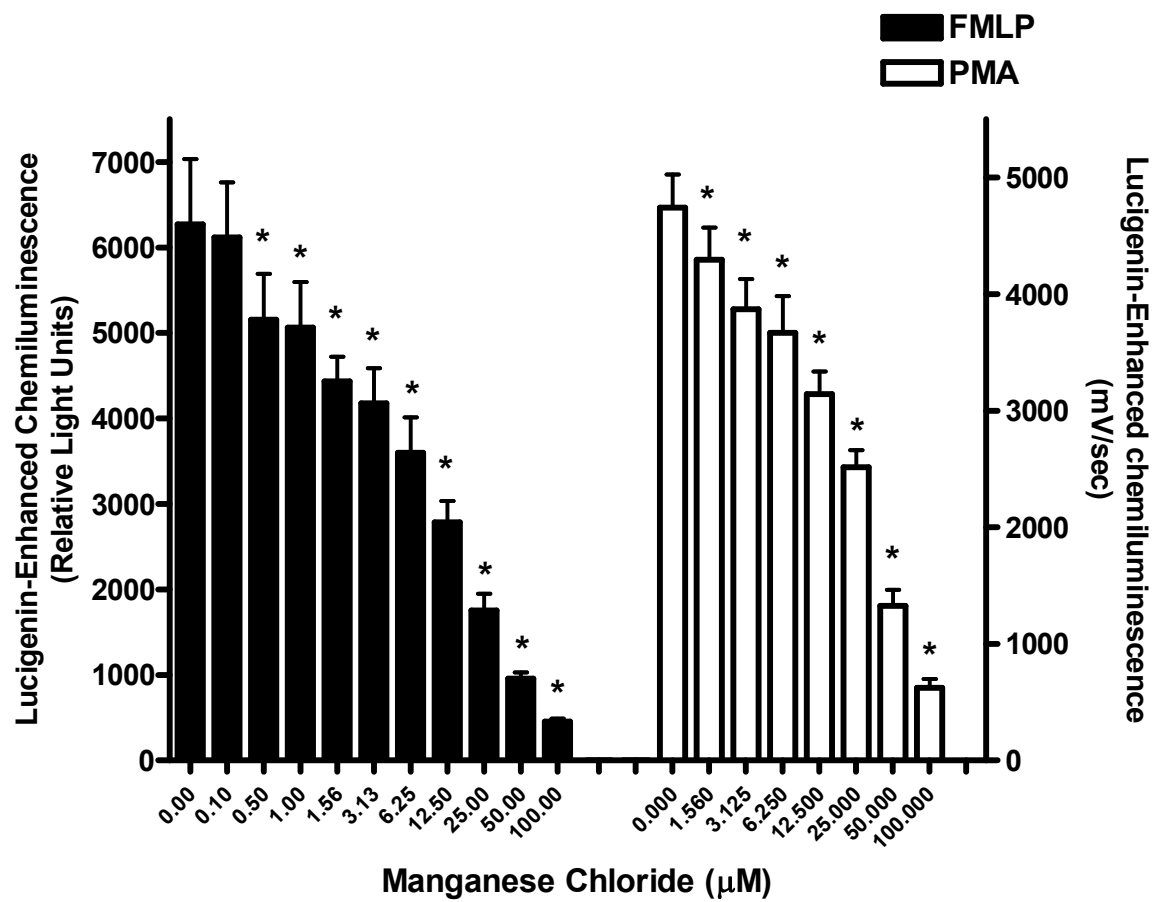


Figure 2

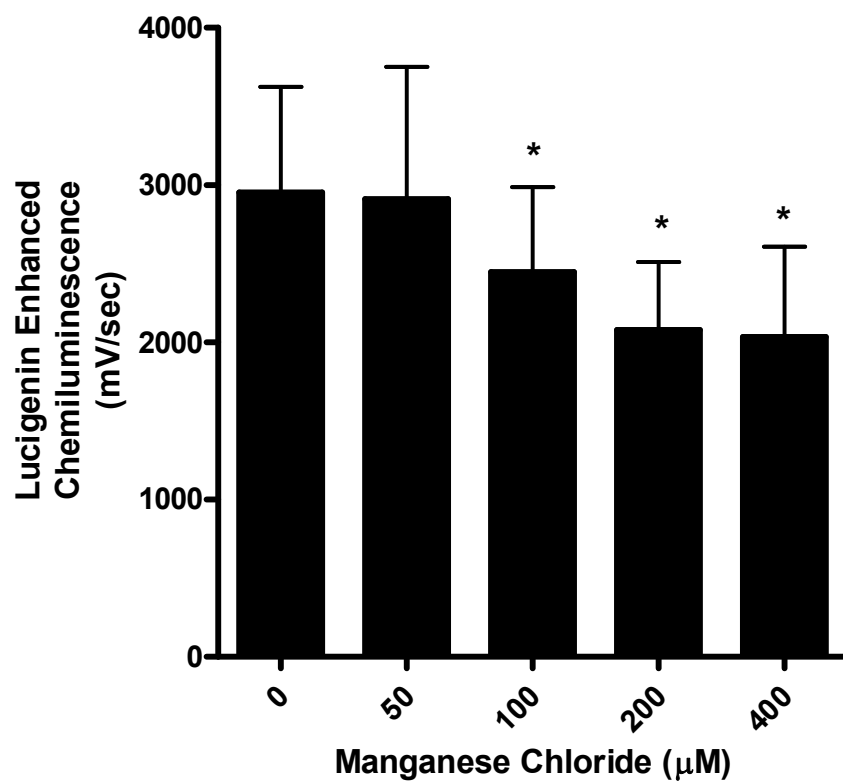


Figure 3

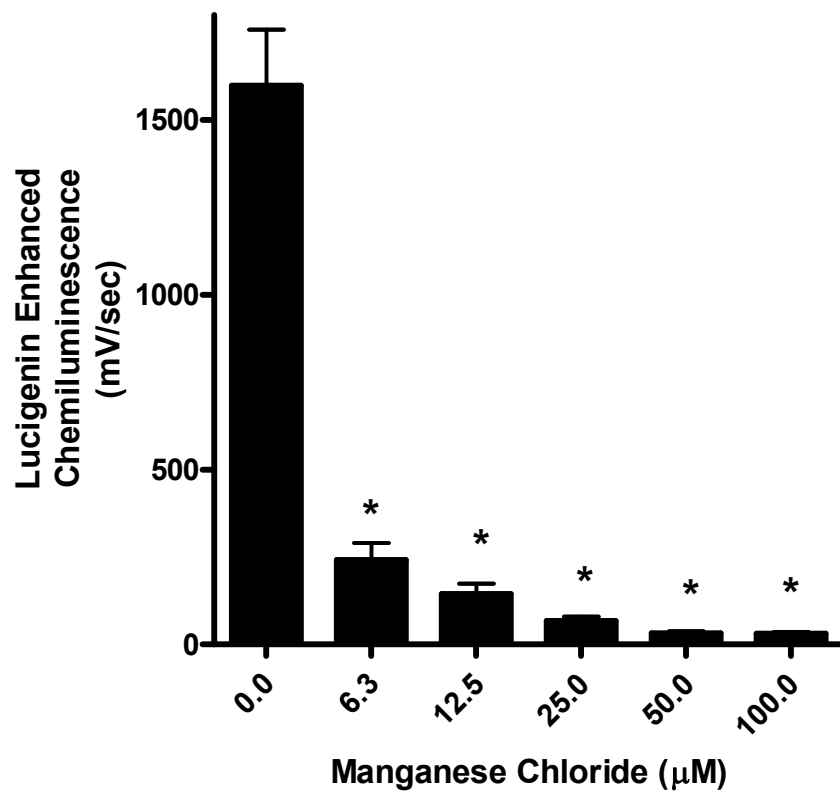


Figure 4

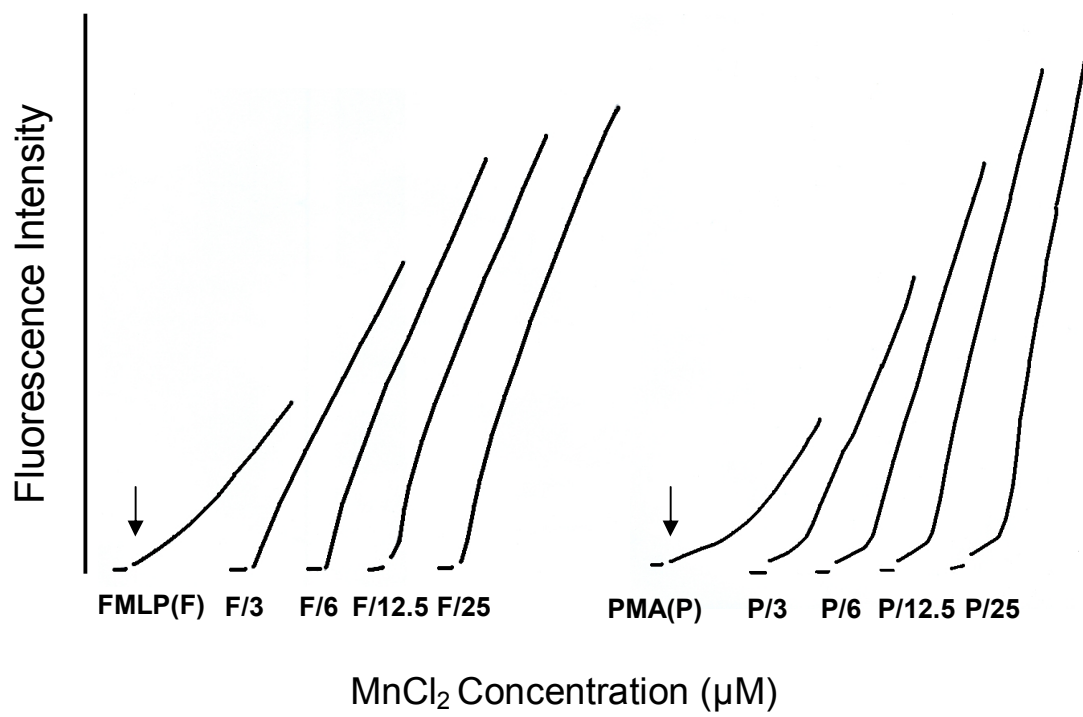


Figure 5

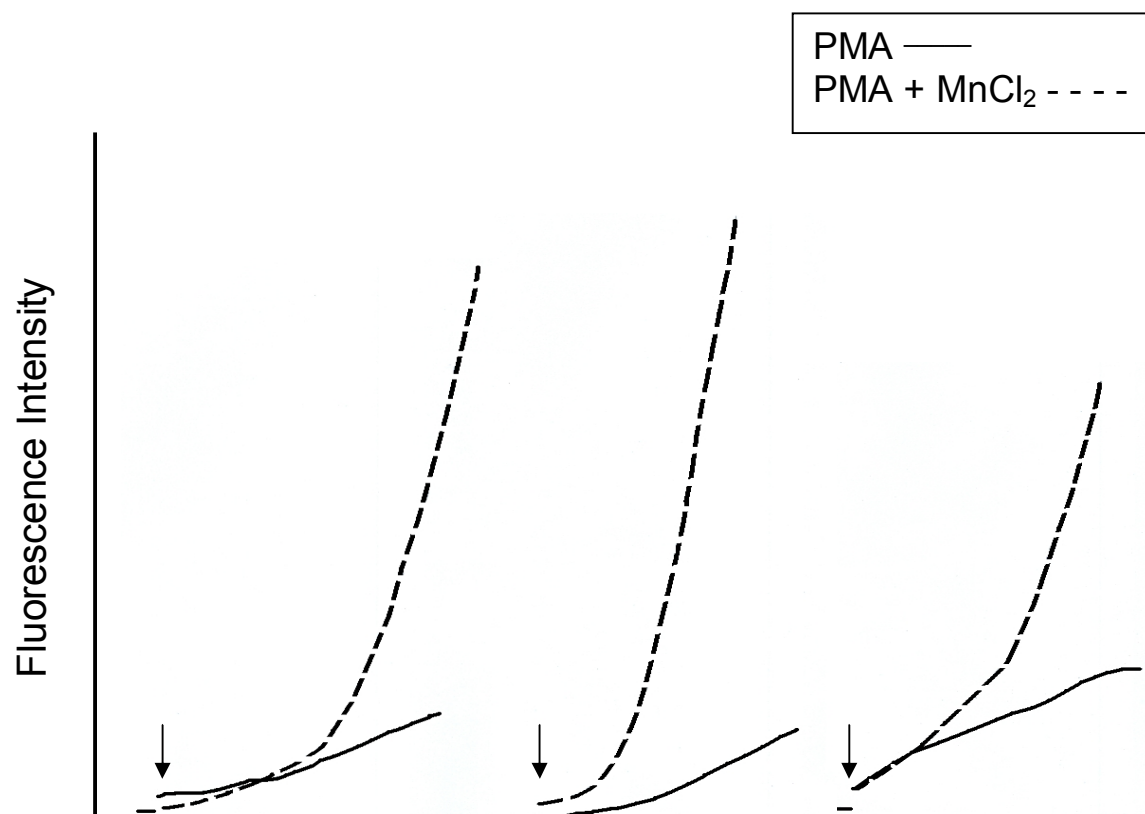


Figure 6

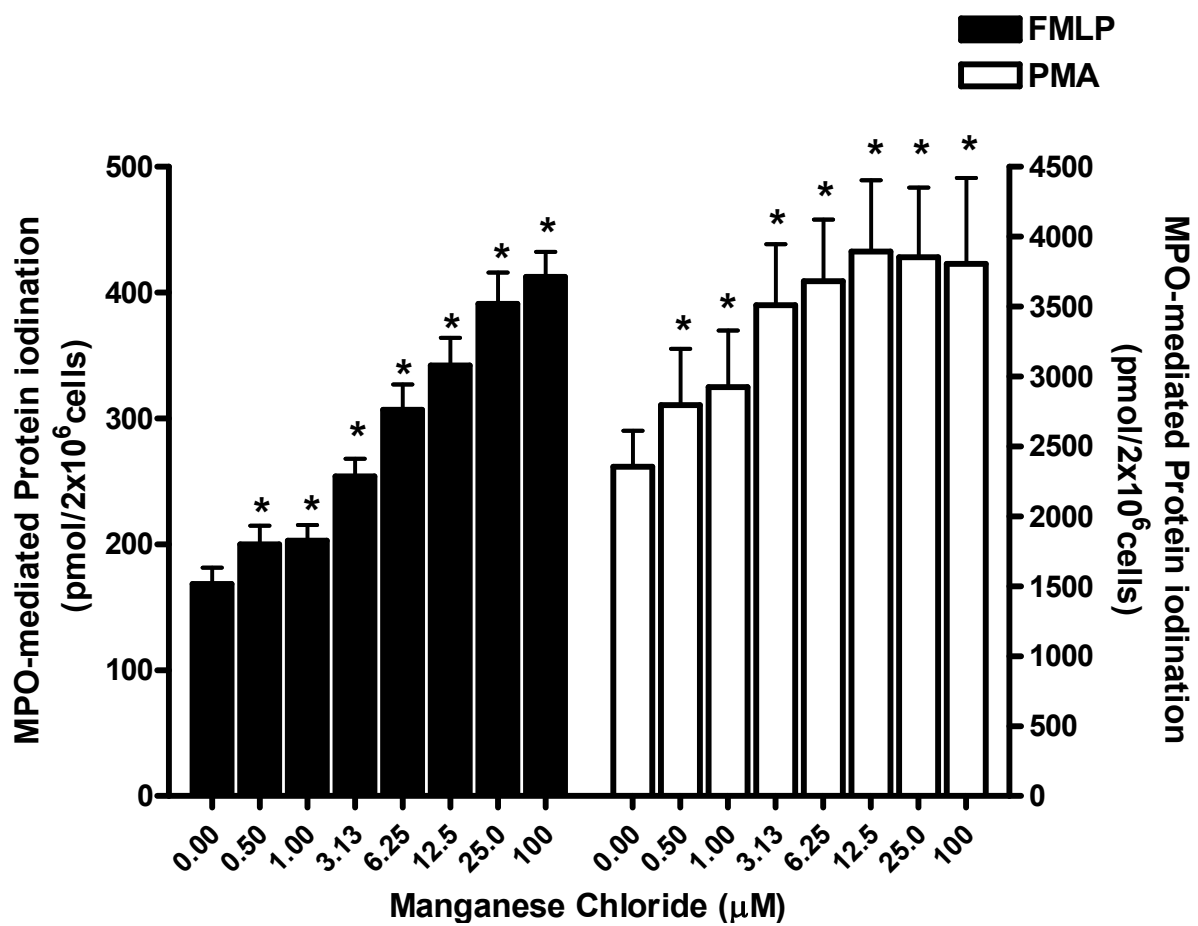


Figure 7

